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Dynamics of success and failure in phage and antibiotic therapy in experimental infections

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Abstract

Background: In 1982 Smith and Huggins showed that bacteriophages could be at least as effective as antibiotics in preventing mortality from experimental infections with a capsulated E. coli (K1) in mice. Phages that required the K1 capsule for infection were more effective than phages that did not require this capsule, but the efficacies of phages and antibiotics in preventing mortality both declined with time between infection and treatment, becoming virtually ineffective within 16 hours.

Results: We develop quantitative microbiological procedures that (1) explore the in vivo processes responsible for the efficacy of phage and antibiotic treatment protocols in experimental infections (the Resistance Competition Assay, or RCA), and (2) survey the therapeutic potential of phages in vitro (the Phage Replication Assay or PRA). We illustrate the application and utility of these methods in a repetition of Smith and Huggins' experiments, using the E. coli K1 mouse thigh infection model, and applying treatments of phages or streptomycin.

Conclusions: 1) The Smith and Huggins phage and antibiotic therapy results are quantitatively and qualitatively robust. (2) Our RCA values reflect the microbiological efficacies of the different phages and of streptomycin in preventing mortality, and reflect the decline in their efficacy with a delay in treatment. These results show specifically that bacteria become refractory to treatment over the term of infection. (3) The K1-specific and non-specific phages had similar replication rates on bacteria grown in broth (based on the PRA), but the K1-specific phage had markedly greater replication rates in mouse serum.

Background

Mounting concerns about drug-resistant pathogenic bacteria [1–3] have rekindled interest in alternative treatments of bacterial infections. Prominent among these alternatives is phage therapy, the use of bacteriophages to kill or otherwise control the bacterial populations in infected hosts. The use of bacteriophage for the treatment of bacterial infections is an old idea [4] that not only caught the imagination of at least one novelist [5] it was practiced with sporadic successes worldwide in the 1920s and 1930s. However, following the development of antibiotics in the 1940s, the use of phages to treat and prevent infections disappeared from so-called Western Medicine, but it did survive in the former Soviet Union. A rekindled

interest in phage therapy over the past decade has inspired historical reviews, increased our awareness of a substantial body of phage therapy work from Eastern Europe [6–8], resulted in the West's "discovery" of the Eliava Institute (a one-time vast and thriving phage therapy research and production facility in Tiblisi, Georgia [9]), and motivated the formation of companies developing phage therapy (Biophage Inc. of Montreal, Canada; Exponential Biotherapies Inc. of Port Washington, NY; Intralytix of Baltimore, MD). In recent years this renewed interest in phage therapy has been displayed in articles in the popular press, (e.g., Kuchment 2001, Superbug Killers, News Week, Dec. 17, 2001 50–51) and, of course, reviews and discussions on the internet, [http://www.phage.org]; [http://www.evergreen.edu/user/T4/PhageTherapy/Phagethea.html].

Renewed interest in phage therapy is also evident from recent empirical studies of phage therapy [10–15] and theoretical excursions into the population dynamics of phage therapy [16,17]. This rebirth has also revived attention to earlier experimental work on phage therapy and prophylaxis, including several impressive studies using phage to treat and prevent bacterial infections in mice, calves, piglets and lambs by H. Williams Smith, M.B Huggins and their colleagues [18–20].

The earliest of the Smith and Huggins studies [18] is especially instructive in this light. Using experimental, lethal infections of an *E. coli* 018:K1:H7 into the mouse thigh, it showed: (i) phage were at least as effective as antibiotics in preventing mouse mortality; (ii) not all phages were equally useful, rather those that required the K1 antigen for infection were superior to phages not requiring K1; (iii) survival declined with a delay in treatment, even though the treatment was applied well before untreated mice had overt symptoms of the infection. Perhaps most importantly, Smith and Huggins [18] also provided information about the dynamics of phage treatment – the changes in bacterial and phage densities over the course of the infection. Ultimately, the efficacy of antibacterial therapy has to be measured by the rate at which it eliminates the symptoms of the infection. Microbiological data on the course of infections with and without treatment – the population dynamics of the treatment process – provide a means to understand how treatment operates and allows one to compare, modify and improve therapeutic protocols.

The present study is offered in the spirit of continuing the precedent set by Smith and Huggins for analyzing the population dynamics of phage and antibiotic therapy. We use their infection model to develop and illustrate the use of two quantitative methods that facilitate understanding, comparing and developing methods of antibacterial therapy and protocols for their application. One method provides a facile measure of efficacy of treatment that is independent of the clinical outcome of infection and can be applied on a tissue- and time-specific basis for any form of treatment to which bacteria can acquire resistance. The other method is a simple procedure to assay phage growth rate in vitro that can be used to screen phages to evaluate their potential for therapy.

Results

Smith and Huggin's results are repeatable

We repeated the experiments of H. William Smith and M.N. Huggins [18]. Despite the fact that we used different sources of mice, *E. coli* 018:K1:H7, bacteria and phages, our treatment and mortality rate results were similar to theirs both qualitatively and quantitatively (Fig. 1):

(a) All 15 untreated/control mice died within 40 hours of inoculation with CAB1. All 15 mice survived when treated immediately with ϕLH (the phage requiring the bacterial K1 antigen for adsorption) or when treated immediately with a single dose of 60 μg/gm streptomycin. In contrast, only 6 of the 15 mice survived when treated immediately with ϕLW (the phage that was not specific for the K1-capsule). These results closely match those of Smith and Huggins [18], except that they did not report experiments of immediate treatment with streptomycin or other antibiotic.

(b) Even though mice with untreated infections normally survived at least 24 hours, delaying treatment for only 8 hours significantly reduced the rate of survival of mice treated with a single dose of 60 μ/gm and of 100 μ/gm streptomycin. The survival rate of these streptomycin-treated mice, however, significantly exceeded that of the controls and that which Smith and Huggins [18] obtained with a single dose of 25 μ/gm streptomycin. The survival of mice treated with ϕLH was slightly reduced with delayed treatment (11 of the 12 mice treated at 8 hours survived, versus 15 out of 15 with immediate treatment), a difference that is not statistically significant. In the Smith and Huggins study, the decline in survival at 8 hours was significant for the combined samples of 9 isolates of K1-specific phages, but not for their most efficacious phage. We did not conduct delayed treatment experiments with ϕLW or with multiple doses of streptomycin.

The Resistance Competition Assay (RCA)

Derivation

The principle of this method is that, when a mixture of resistant and sensitive cells is treated, the relative frequency of bacteria resistant to treatment will increase at a rate according to efficacy of the treatment. That is, resistant cells increase relative to sensitive cells by the amount that treatment kills or inhibits sensitive bacteria. The derivation of our measure of this efficacy of treatment, *RCA*, follows

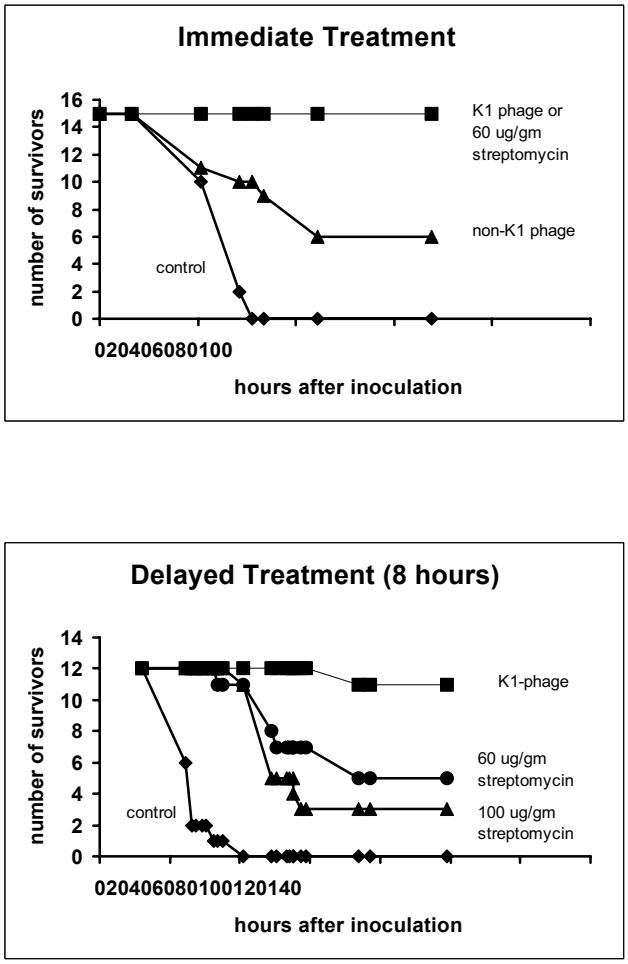

Figure 1
Survival of CAB1 infected mice: (Top) Immediate Treatment – Mice were inoculated with 10^8 CAB1 in the right thigh and within one minute treated by inoculation in the left thigh with (i) a control (saline or a CAB1-pseudolysate) (ii) 10^8 non-K1 specific phage ϕLW, (iii) 10^8 K1-specific phage ϕLH, or (iv) 60 μg/ml streptomycin. (Bottom) Delayed Treatment: – Mice were inoculated with 10^8 CAB1 in the right thigh and, 8 hours later, treated by inoculation in the left thigh with (i) 0.85% saline (control), (ii) 10^8 K1-specific, ϕLH, phage, (iii) 60 μg/gm streptomycin, or (iv) 100 μg/ml streptomycin.

from standard population genetics models of selection between two haploid genotypes that different in their relative fitness [21]. (Following convention, we use italicised *RCA* for the parameter value or estimate, and normal font RCA for the assay procedure.) In this perspective, sensitive and resistant bacteria are the two competing genotypes. The RCA estimates the relative fitness advantage (or disadvantage) of the resistant bacteria over sensitive bacteria. In situations where one has the luxury of many sampling points, an *RCA* can be calculated from the slope of the natural log of the ratio of the densities of Resistant/Sensitive as a function of time [22]. In situations where only two points are feasible, the initial frequency of resistant bacteria in the inoculum (p_0) and the frequency of resistant bacteria at time t (p_t) an *RCA* can be calculated from

$$RCA = \ln [(p_t (1-p_0))/(p_0(1-p_t))]/t \quad (1)$$

(for the derivation see Crow and Kimura [21]).

The value of *RCA* is the selection coefficient of resistant cells (in the vernacular of population genetics). If $RCA > 0$ then the resistant cells have an advantage, if $RCA < 0$, the resistant cells are at a selective disadvantage. *RCA* operates on an exponential scale:

$$p_t/(1-p_t) = e^{tRCA} (p_0/(1-p_0). \quad (2)$$

If the differential success of sensitive and resistant cells is constant over time, then the ratio of resistant to sensitive cells increases by a factor of e^{RCA} every hour (unit of t). Assuming that cells are not growing and that treatment kills sensitive cells, an *RCA* of 0 means that sensitive cells are not killed, an *RCA* of 1 means that 63% of sensitive cells are killed per hour (95% in 3 hours), and an *RCA* of 2 means that 86.5% of sensitive cells are killed per hour (99.8% in 3 hours). If the process is not constant, then the *RCA* is an average over the interval of estimation and should not be projected to longer intervals; however, estimations of *RCA* at different times would reveal how treatment efficacy is changing with time.

As an alternative to using the initial frequency of resistant cells as the estimate of p_0, one can substitute the final frequency (q_t) of resistant bacteria in untreated controls (as was done here). This substitution controls for intrinsic differences in fitness of the two bacteria, for differences in the physiological states of the bacteria at the time of inoculation that could affect their growth rates independent of treatment, and for possible differences in the host response with and without treatment. The resulting *RCA* is thus the advantage of resistant cells over sensitive cells relative to their advantage in the absent of treatment. For example, in the absence of antibiotic treatment, resistant bacteria may have a disadvantage in competition with sensitives [23], and use of q_t in place of p_0 will correct for this effect. It is important, however, that the controls from which q_t is obtained be inoculated with the same mix of bacteria and be sampled at the same times as the treated mice.

Bacteria become refractory to phage and antibiotics within 8 hours
As noted in Figure 1 and in more detail in [18–20], the survival rate from treatment with phage and antibiotics declines dramatically with the term of infection. A declining efficacy of delayed treatment is also evident by com-

Table 1: Resistance Competition Assay comparison of treatment factors

	RCA value	
Treatment	**Immediate**	**Delayed**
ϕLH-Phage	1.7 ± 0.14	0.3 ± 0.13
streptomycin	2.1 ± 0.12	0.5 ± 0.10
ϕLW-Phage	0.2 ± 0.07	not assayed

Resistance Competition Assay (RCA) of bacteria from the infected legs of mice sampled 3–4.5 hours after treatment of CAB1 infections (mean ± 1 std error). Bacterial resistance was specific to the treatment, phage or streptomycin. Immediate treatment was delivered at the same time as the bacterial inoculation; delayed treatment was delivered 8 hours after inoculation. For treatment with streptomycin and ϕLH-phage, resistant bacteria have significantly higher *RCA* values with immediate treatment than with delayed treatment (t-tests, $P < 0.002$ for ϕLH-phage; $P < 0.005$ for streptomycin). With immediate treatment, *RCA* values of resistant bacteria are significantly higher both for streptomycin and ϕLH than for ϕLW ($P < 0.005$ and $P < 0.01$, respectively), but the difference between immediate treatment with streptomycin and ϕLH is not significant ($0.05 < P < 0.10$). An *RCA* value of zero indicates that resistant bacteria have no advantage over sensitive bacteria, hence that treatment is presumably ineffective in killing bacteria in the leg; presumed treatment efficacy increases as the *RCA* increases above zero. Treatment doses were ~10^8 phage/mouse or 100 μg/gm streptomycin sulfate via an intramuscular injection into a limb. Frequencies of sensitive and resistant bacteria were estimated as in Methods.

paring *RCA* values for ϕLH or streptomycin (Table 1). The *RCA* for a single dose of streptomycin was 2.1 after immediate treatment but declined to 0.5 when treatment was delayed 8 hours. The corresponding values for the K1-specific phage were 1.7 and 0.3. The *RCA* for ϕLH at 8 hours is statistically indistinguishable from that for immediate treatment with ϕLW (0.2), even though the mouse survival under these two conditions differs significantly (Fig. 1; $P < 0.02$). It is thus evident that by eight hours the bacteria had declined in their susceptibility to phage, and antibiotic efficacy declined as well. It is noteworthy that there were no apparent clinical symptoms of these infections at 8 hours (or even at 12 hours).

The bacteria inoculated into the thigh not only became refractory to antibiotic and phage treatment within a few hours after inoculation, they persisted and remained refractory to phage treatment for a number of days if the mouse survived. In samples taken 7 days after treatment, the density of the ϕLH phage in the thigh was 10^9 phage particles per gram, and while phage resistant cells were present, 80% (0.81 ± 0.01) of the bacteria isolated from leg tissue remained sensitive to this phage.

The Phage Replication Assay: phages ϕLW and ϕLH have similar growth rates in broth but not serum

Mortality rates of mice in Smith and Huggins [18] and here, as well as the RCA values, indicate that phages specific for the K1-capsule are more effective in controlling the infections than phage that are not specific for the capsule. Could this have been anticipated from the in vitro capacity of the phage to replicate on and kill E. coli 018:K1:H7? The Phage Replication Assay (PRA) attempts to provide a measure of in vitro efficacy that can be extended to in vivo performance. The rate of doubling of a population of lytic phage on a population of bacteria measures the ultimate efficacy of that phage in killing those bacteria. This overall replication rate depends on adsorption rate, latent period, and burst size on that bacterial host, as well as temperature and other physical properties of the culture conditions (see Additional file 1). The rate of replication also depends on the ratio of phage to bacteria (the multiplicity of infection) and most critically on the density and physiological state of the bacterial population [24,25]). Despite the many factors that influence phage replication rates (and hence influence the PRA value), these factors are easily controlled, and furthermore, the comparison of PRAs between different phages is straightforward if the PRAs are obtained from common bacterial cultures (as here, see Methods).

Our *PRA* values are surprising in some respects yet are consistent with the mouse mortalities and microbiological data. When the *PRA* of these phage were estimated on cells grown in Luria Broth, there was no apparent difference between the two phages (Fig. 2); the *PRA*s were strongly affected by cell densities, but both phages showed similar patterns, and there is no basis from these in vitro data for suggesting any difference in their efficacy at replication on and killing CAB1. Note that the observed *PRA*s declined with increasing bacterial density, the opposite of the effect seen in the simulations of Additional file 1. There are in fact two opposing effects of cell density on the PRA. One is that higher cell densities reduce phage generation time by decreasing the time for a phage to encounter a host. This increases the *PRA*. The other is that high cell densities exhaust nutrients in the media and thereby slow bacterial growth, in turn reducing phage metabolism and thus reducing the *PRA*. Only the former effect was considered in the model of Additional file 1.

When the *PRA* was estimated on cells grown in mouse serum, the K1-specific phage ϕLH had a much higher rate of replication than ϕLW (Fig. 2). Despite variance in the estimates, there was a pronounced superiority of the K1-specific phage ϕLH over the non-specific phage ϕLW. Although this serum assay did not fully mimic in vivo growth conditions, it certainly supports the hypothesis that the greater treatment efficacy of ϕLH is specific to

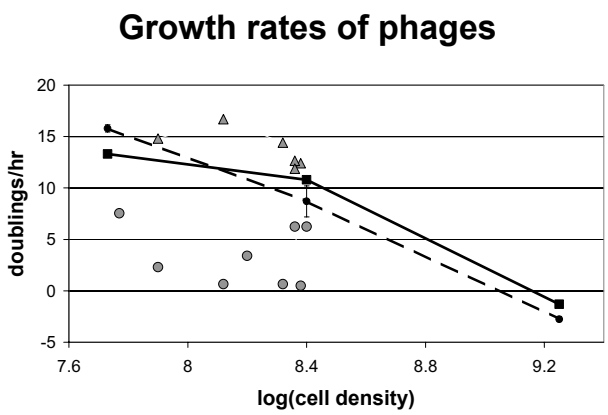

Figure 2
Phage Replication Assay: Rate of replication of ϕLH and ϕLW on CAB1 in LB and in mouse serum. In LB, the growth rates of the K1 phage (ϕLH, squares connected by the solid line) are similar to those of the non-K1 phage (ϕLW, solid circles connected by the dashed line) across a range of densities, with both phages growing more rapidly on cells at low density than on cells at high density, near saturation. However, on cells grown in mouse serum, the growth rate is much higher for the K1-specific phage, ϕLH, (gray triangles) than for the non-K1 specific phage, ϕLW (gray circles). Growth rate is presented as doublings of phage concentration per hour; standard error bars are given for each black point (on the lines), but most are so short as to be obscured by the point. Each gray point represents one assay in serum.

phage replication on bacteria in mice and is not from a general superiority of ϕLH.

Discussion

Our goal here was to develop procedures to explore the dynamical underpinnings of success and failure in treating bacterial infections. To illustrate the application of these procedures, we used the *E. coli* K1 mouse thigh infection model of Smith and Huggins [18], and like them, applied treatments of phages and antibiotics. As part of this effort, we repeated their experiments to ascertain if their results were robust. Across a variety of treatments and conditions, mouse mortality rates were remarkably similar between our study and theirs, even though we used a different strain of mice, phages from different sources, and an independently isolated strain of *E. coli* 018:K1:H7. Thus, in the absence of treatment, inoculations of 10^8 bacteria were fatal in over 95% of mice, but immediate treatment with phage that were specific for the K1 capsule or with streptomycin essentially eliminated mortality from the infection. Immediate treatment with phages that were not specific for the capsule also reduced mortality, but mortality rates for treatment with these phage were greater than with the K1-specific phage. Both studies also observed that mortality increased if treatment was delayed by 8 hours, under most forms of treatment.

Factors affecting treatment success

Two observations from these combined studies seem especially interesting. (i) Phages vary in treatment success. (ii) Delaying treatment by 8 hours substantially increases the rate of mortality relative to that of immediate treatment (for many of the treatments applied here), despite the fact infected mice survive at least 16 hours longer. In attempting to better understand these observations, we developed two assays. One assay, the RCA (**R**esistance **C**ompetition **A**ssay) measures the efficacy of a treatment in killing or inhibiting the growth of bacteria based on changes in the frequency of treatment-resistant bacteria after treatment is applied. The other assay, the PRA (**P**hage **R**eplication **A**ssay), measures the ability of a phage to replicate on populations of a bacterium under controlled, in vitro conditions.

The *RCA* and *PRA* values reflect what is observed with other (microbiological) measures of the efficacy of treatment. Consider first the effect of delayed treatment, which reduced mouse survival in most treatments. Increased mortality with delayed treatment could have various causes: (i) By the time of treatment, the numbers of bacteria are already sufficiently high that mortality is caused by toxins and tissue damage ensuing from them, rather than from further growth of the bacteria. Under these conditions, reducing the number of bacteria by treatment would have little effect on the clinical outcome of the infection. (ii) By the time of treatment, the numbers of bacteria are so high that the mouse's defenses cannot prevent further growth of the bacterial population, even with treatment, and bacterial numbers simply increase to the point of mortality. This model is similar to that in (i), except that a treatment which controlled bacterial growth would prevent mortality. (iii) As a consequence of the host's response to the proliferating population of bacteria, the site of the infection becomes less accessible to the antibiotic or phages, and the proliferation of the bacterial population can no longer be controlled by these antibacterial treatments. (iv) The bacteria become physiologically refractory to the treatment.

The RCA specifically supports the latter two models. If by eight hours the bacteria were as accessible and responsive to the phage or antibiotic as they were initially, the *RCA* values would remain high. We instead observed a drop in *RCA* values. The data do not rule out additional processes consistent with models (i) or (ii), but the data clearly reveal a reduced bacterial susceptibility or reduced access of treatment to the bacteria when treatment is delayed.

More than a half a century ago, H. Eagle and colleagues observed a dramatic decline in the efficacy of antibiotics with the term of the infection in a mouse thigh model. In their now classical investigations of the within-host dynamics of antibiotic treatment, they followed the course of penicillin treatment of *Treponema pallidum* and Group A and Group B Streptococcus [26–28]. Eagle [28] postulated that the most likely reason for the declining efficacy of treatment with the term of the infection was due to (i) a decline in the rate of metabolism and replication of bacteria during the infection, and (ii) that slowly growing or non-growing bacteria are more refractory to penicillin than those that replicating at higher rates. More recent work by E. Tuomanen, A. Tomaz and their colleagues supported this interpretation, a phenomenon they called phenotypic tolerance [29–31]. They also provided evidence that nutrient limitation was the cause of the decline in the rate of bacterial growth [32]. Not only do bacteria become increasingly refractory to the majority of antibiotics as their rate of growth declines, but they also adsorb to and replicate bacteriophage less efficiently [24,33], as is evident from the *PRA* values in Fig. 2. Thus the decline in the efficacy of phage and antibiotic therapy with delayed treatment in our experiments is plausibly attributed to a decline in the rate of replication of *E. coli* K1 once inside the mouse.

The other intriguing observation from Smith and Huggins that we corroborated here is the consistent superiority of phages requiring the K1 antigen for infection, when measured as mouse survival. Our *RCA* values showed a significant difference between the phages in vivo, suggesting that the K1-specific phages replicate at a higher rate than the non-K1-specific phages inside the mouse. Yet although Smith and Huggins casually observed that their non-K1-specific phages were inferior to K1-specific phages at lysing cultures of bacteria grown in artificial media, suggesting intrinsic differences between the phages more generally, our *PRA* estimates indicated that there was no intrinsic difference between our two phages in artificial media. Instead *PRA* differences were consistent with mouse survival and *RCA* data only when the *PRA* was measured on cells grown in mouse serum. Thus the *PRA* enables one to begin unravelling the environmental bases of differences in phage growth in vivo.

In this investigation, we used the mouse thigh infection model because of the many precedents for its use and because of its established repeatability [34–37]. The fact that bacteria undergo such a profound change in susceptibility or accessibility to treatment in only 8 hours raises the question of whether the mouse thigh infection model is representative of natural infections. This question is not necessarily answerable at present, but the results do highlight the fact that development of specific protocols and phages for treatment in any one experimental model may be inadequate for treatment of the same bacterium under field conditions.

The Resistance Competition Assay

The preceding discussion indicates that the resistance competition assay is consistent with other measures of phage performance in vivo and provides specific insights not easily obtained in other ways. The RCA has other virtues that make it useful for studying phage therapy and other forms of treatment. (1) It is versatile. In addition to being used to study the population dynamics of phage and antibiotic treatment in general, the RCA could be employed to design and evaluate the efficacy of different antibiotic treatment protocols. It can be applied to virtually any experimental model, such as enteric infections [38,39] or urinary tract infections [40]. (2) The RCA provides a more direct measure of the in vivo action of antibiotics (or other treatments) than estimates of the concentrations of these compounds in serum or in solid tissue. Moreover, the RCA protocol controls for the contribution of the host defenses as well as variety other factors that could influence the density of bacteria in a particular tissue. Only the action of the treatment per se can account for the difference in the frequency of resistant bacteria between treated and untreated treated hosts. (3) The RCA is more humane and offers greater statistical power (per animal) than "outcome" measures of the efficacy of treatment based on survival or other clinical indications. The fact that the RCA yields a continuous statistic, rather than a binary one, enables the use of standard statistical analyses in which meaningful comparisons can be made with as few as two replicates per group. (4) The RCA can be applied to populations of bacteria infecting specific tissues at specific times, even allowing multiple measures per animal. However, individual tissues subjected to the RCA must not have high levels of bacterial migration from other tissues over the course of treatment.

The RCA is a sensitive and specific measure of treatment efficacy, and as such, will not correlate perfectly with other measures of treatment. To wit, our *RCA*s were similar between delayed ϕLH treatment (0.3) and immediate ϕLW treatment (0.2), even though mouse survival rates were significantly different between them (11 of 12 mice versus 6 of 15). These discrepancies highlight the complexity of the infection process and the fact that different measures of infection dynamics capture different properties. Because our *RCA*s were based on samples from the infected thigh, the values apply specifically to that thigh and do not necessarily reflect the efficacy of treatment in other tissues that may influence host survival.

The Phage replication assay

This method has a potential utility beyond that demonstrated here. If phage therapy is to be developed for particular infections, it would be useful to have an in vitro procedure to screen phages for their potential efficacy in vivo. The diversity of phage is enormous. For example among 40 phage isolated from different samples or different plaques on lawns of *E. coli* K12 and *E. coli* B, at least 32 distinct phage were found [41] (as measured by host range and/or restriction pattern). A far greater number of phage could certainly be isolated with a broader array of lawn bacteria and by sampling different sources. The implication is that it should be possible to isolate a substantial number of lytic phage capable of killing most strains of enteric and other bacteria, hence offering a compelling reason to pursue phage therapy as a solution to antibiotic resistance. Screening many phages for their therapeutic potential in experimental animals would be a time- and animal-consuming task. The PRA employed here could facilitate that screening. Our results suggest that phage performance in serum could be a sufficient indicator of in vivo performance, but there is no reason that this assay could not be performed in vitro with other modifications, such as solid tissues. Moreover, as suggested in a recent study [12], in vivo culture may selectively improve the capacity of a phage to replicate on and kill bacteria in a mammalian host.

Prospects for phage therapy

This is a methodological study to develop and experimentally evaluate procedures to measure the efficacy of antibacterial treatment and to screen bacteriophage for their therapeutic potential. Our purpose in performing these experiments and publishing these results is not to advocate the use of phages for the treatment of systemic infections. Nevertheless, because of the current novelty of and aspirations for phage therapy as an alternative to antibiotics, it seems appropriate to acknowledge that our results and those of several other studies offer promise that phage therapy is highly repeatable, can be successful, and is thus worthy of further research for clinical practice. Moreover the use of phage for therapy and prophylaxis needn't be restricted to humans, as phage could obviously be used for these purposes in domestic animals.

There are, of course, a number of problems associated with the use of phage as an alternative to antibiotics. To us the most serious biological problem is a restricted host range. Not only would one have to know the species of bacteria responsible for an infection, it would be necessary to know which phages can infect that strain of bacteria. These requirements are certainly inconsistent with current empiric therapy that uses broad spectrum antibiotics, which dominates how antibiotics are employed in the community as well as in hospitals. Commonly, it is not clear whether a bacterial infection is responsible for the symptoms being treated with antibiotics, much less the species, strain, and resistance profile of the bacteria responsible. On the other hand, there are situations, like epidemics, where this knowledge would be available. And as procedures to identify the bacteria responsible for symptomatic infections get better and more rapid, it soon may be quite easy to get this information from individual patients. As a consequence of the ever increasing frequency of antibiotic resistant bacteria, the range of antibiotics to which individual bacteria are resistant, and the limited number of targets to which current (and soon to be anticipated) antibiotics are directed, there is a pressing need to develop alternative methods of treating and preventing bacterial infections. Phages certainly offer some of the most readily-available and promising alternatives.

Conclusions

(1) The results of the Smith and Huggins 1982 study of phage and antibiotic therapy [18] are repeatable and robust quantitatively as well as qualitatively. Using their almost invariably (more than 95%) lethal *E. coli* K1 mouse thigh infection protocol, but with different strains of mice and independently isolated phages and bacteria, we obtained the same frequencies of treatment survival as they did. As they also observed, phages that required the K1 capsule for infection provided greater mouse protection than phages that did not require K1. When treatment was administered within minutes of the infection, both the K1 specific phage and single doses of streptomycin completely prevented infection-induced mortality. If, however, treatment was delayed by eight hours, the rate of recovery with a single dose of streptomycin (the most effective antibiotic in the Smith and Huggins study) was reduced by approximately 50%.

2) The Resistance Competition Assay (RCA), one of two protocols developed here, uses the change in the frequency of a minority population of bacteria resistant to the treating agent as a measure of the efficacy of treatment in vivo. Our *RCA* estimates from phage and streptomycin treatment of *E. coli* K1 infections in laboratory mice are consistent with mortality rates. With immediate treatment, the *RCA* values for the streptomycin and K1-specific phages (which totally prevented mortality) were both substantially greater than that of the phage not specific for the K1 capsule (for which 60% of the treated mice died). *RCA* values for the K1-specific phage and for single doses of streptomycin when treatment was delayed by eight hours were substantially and significantly less than those estimated for immediate treatment. The RCA supports earlier observations that as time between infection and treatment increases, bacteria become increasingly refractory to treatment (e.g., because of changes in bacterial physiological and/or the host environment).

3) The Phage Replication Assay (PRA) provides a simple measure of the rate of replication (and host cell killing) of lytic phage under defined conditions. By varying those conditions, it is possible to identify factors that affect phage growth and hence their efficacy in killing the target bacteria. Over a wide range of densities of bacteria growing in artificial medium, the estimated *PRA*s were similar between the K1-specific and non-specific phages. Based on these estimates we would not have anticipated different efficacies of these phage in treating E. coli K1 infections. On the other hand, the assay performed in mouse serum yielded substantially greater efficacy of the K1-specific phage, matching its superior performance in the mouse.

4) The results of this investigation, like those of Smith and Huggins and others, support the potential of phage for treating bacterial infections and the development of experimental infection models to evaluate and optimize the efficacy of antibiotic as well as phage treatment protocols. Our results illustrate the value of exploring the dynamics of the bacterial population during treatment in the evaluation and design of treatment protocols

Methods

Culture and sampling medium and procedures

In vitro, liquid cultures of bacteria and phage were grown and maintained in Luria – Bertani broth (LB), supplemented with 1 gm/l glucose. Bacterial densities were estimated from colony counts on Petri dishes (plates) containing 25 mL of LB with 1.75% agar. Phage densities were estimated on these plates with 3 ml top (0.7%) agar containing LB glucose and 100 ul of an overnight culture of the bacteria (about 2×10^9 bacteria per ml). When needed, the bacterial and phage suspensions were serially diluted in 0.85% saline or LB Glucose before plating. When rare (less than 10%), phage-resistant bacterial densities were estimated by plating with and without $\sim 10^9$ phage particles in soft agar; when common, they were estimated by streaking individual colonies across high densities of phage on plates. A similar selective plating procedure was used to estimate the densities of antibiotic resistant bacteria.

Bacteria

The primary strain used in these experiments was CAB1, an *E. coli* of the same serotype as that used by Smith and Huggins [18] (O18:K1:H7), but isolated from a different source [42]. We also used a TcR, Kps-, *E. coli* K-12 chimera of this isolate, designated CAB281, that does not express the K1 antigen [42].

Phage

Phages were isolated from an Atlanta, Georgia sewage treatment plant. An aliquot of liquid sewage from the inflow to the plant was treated with chloroform to kill bacteria and human viruses. The chloroform-free supernatant was then enriched for *E. coli* 018:K1:H7-specific phage by adding 1 ml of this suspension to 10 ml LB containing CAB1 (approximately 4×10^7 bacteria per ml). These mixtures of bacteria and sewage were grown for a minimum of four hours, mixed with chloroform, and centrifuged to remove debris. The vast majority of phages in these crude lysates were able to grow on CAB281 as well as CAB1, hence were not K1-specific. To enrich for K1-specific phage, the mixed lysate was incubated with CAB281 and treated with chloroform 10 minutes later to abort infections. After removing the chloroform, the cycle was repeated 4 times. The phage were then plated with lawns of CAB1 and single plaques were patched onto lawns of CAB281. Of the 50 plaques isolated after this passage, two were from phage that grew on CAB1 but not on CAB281, hence were presumed to be specific for the K1-capsule. From these plates, one clone each of a K1-specific and non-K1 specific phage was chosen, designated ϕLH and ϕLW, respectively.

The two phages were characterized morphologically and molecularly. Electron micrographs revealed that both had a B1 morphology [43] similar to phage λ but with a tail of hexagonal symmetry and a somewhat shorter head-tail connector than λ. Whole genomes of both phages migrated in agarose gels at approximately 40 kb; genomes of ϕLH and ϕLW were sensitive to *Xmn* I digestion but showed different digestion patterns.

In vivo experiments

All mouse experiments were conducted at Emory University using protocols approved by the Emory University Institutional Animal Care Committee. All of the *in vivo* experiments were performed with female, outbred, white (Swiss) mice (Harlin Sprague) of from 6 to 10 weeks of age (22–30 grams). Mice were maintained in cages containing 5 or fewer animals. Using 1 ml tuberculin syringes, suspensions of bacteria were injected into a mouse thigh. For treatment, phage lysates, pseudolysates (phage-free bacterial cultures treated with chloroform), saline or streptomycin sulfate (SIGMA™) solutions were injected into the opposite thigh or a forelimb muscle. Although chloroform was absent from injected lysates and pseudolysates, no other attempts were made to refine these preparations. Except as noted, the numbers of bacteria and phage injected were each approximately 10^8 and the total volume injected was normally 0.1 ml or less. Following the initial inoculation, the infected animals were periodically observed (usually at intervals of less than 12 hours), with more frequent observations (2 hour intervals) made during the 28–40 hour post infection period when mortality was anticipated. Infected mice surviving beyond 48 hours never succumbed to the infection in the

next 5 days. In situations where the mouse's ataxic appearance indicated that death was imminent, the mouse was euthanized.

Samples of bacteria and phage taken from leg muscle were maintained for a maximum of 1 hour on ice before being weighed and suspended in 2 ml of saline and homogenized with a Tissue Tearor™. The densities of bacteria and phage in these homogenates were estimated by diluting and plating.

Resistance Competition Assay (RCA)

This assay measures the efficacy of phage or streptomycin in limiting replication by an infecting population of bacteria in infected mice. The principle underlying this assay is that bacteria resistant to a treatment will increase in frequency over sensitive bacteria only to the extent that the treatment is effective at killing or reducing the rate of growth of the sensitive bacteria. The assay converts the advantage of resistance into a measure of treatment efficacy. For this assay, mice were inoculated with mixtures of 10^8 bacteria (CAB1) that were sensitive to the treatment (phage or antibiotics) along with low frequencies (10^{-2}–10^{-3}) of bacteria resistant to the treatment. The mice were then treated with phage or streptomycin either immediately (0 hours) or after a delay of 8 hours, or were inoculated with sterile 0.85% saline (controls). At 3, 4, or 4.5 hours after treatment, the mice were sacrificed and the relative frequencies resistant bacteria in the mixtures were estimated from the homogenized leg tissue. We applied this assay in two ways: 1) By using different mice for the immediate and delayed treatments or, 2) by inoculating the same mouse in different hind limb thighs, with the second inoculation delayed 8 hours, and treating the mouse immediately by inoculation of streptomycin, phage or saline into the musculature of a forelimb

Phage Replication Assay (PRA)

This procedure estimates in vitro the potential therapeutic efficacy of phages. It simply measures the rate at which a phage population increases in density (its rate of replication) when grown on a strain of bacteria under prescribed, controlled conditions. Phage replication in vivo is presumed to be an integral part of phage therapy success, so to the extent that replication in vitro mirrors replication in vivo, this assay should indicate which phages are the best choices for therapy. The rate of replication of ϕLH and ϕLW on CAB1 was measured in LB and in mouse serum. Assays in LB were conducted as follows. The bacteria were grown with aeration in LB at 37° until reaching a specified density as measured by light scattering from a side-arm flask. Glucose and calcium were not added to the LB in these assays. Aliquots of 1 ml of the growing bacterial cultures were added to empty tubes along with the phage. The suspensions were grown for 1 hr at which time chloroform was added. Assays in mouse serum were conducted similarly, except that the densities at the time the phage were added were determined by plating and thus could not be standardized between replicates. The bacteria were allowed to replicate in serum for at least 2 hours before addition of phage. Phage concentrations were determined at the beginning and end points to calculate a per-capita increase ratio, ρ; we then transformed this value to $\log_2(\rho)$ to estimate of the number of doublings per hour (see the Additional file 1 for the sensitivity of this measure).

Several precautions were taken to avoid biases and reduce the error in estimating ρ. Phage concentrations were maintained at low levels throughout the assay so that uninfected cells did not become limiting (the final phage density was never greater than the cell density, indicating that a majority of cells remained uninfected during the assay period). To reduce sources of variance in growth rate extrinsic to the phages, growth rate assays of both phages were usually conducted simultaneously with aliquots of the same parent culture for both phages.

Authors' contributions

JB and BL derived the methods used in this paper and conducted the experiments reported in Fig. 1; BL created the Additional file 1. JB conducted the PRA measures in Fig. 2. BL, NW, and TD carried out the RCA assays of Table 1. CB provided the bacterial strains CAB1 and CAB281 and advice. All authors approved the final manuscript.

Additional material

Additional File 1

Sensitivity of Phage Replication Assay to Growth Conditions. This file explains some assumptions that underlie the Phage Replication Assay (PRA), how assay conditions may violate those assumptions and affect those values estimated, and how to minimize the effect of violating those assumptions. A mathematical model using differential equations is provided to evaluate the magnitudes of some of these effects.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2180-2-35-S1.pdf]

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